A rejection under 35 U.S.C. § 101 is improper when a person of ordinary skill in the art would find credible disclosed features or characteristics of the invention, or statements made by the applicant in the written description of the invention. *See,* M.P.E.P. §§ 2107.01(II), (III) at 2100-[29-31] (Rev. 1, Feb. 2000). In addition, an applicant need only make *one* credible assertion of utility for the claimed invention to satisfy 35 U.S.C. § 101. *See, e.g., Raytheon v. Roper,* 724 F.2d 951, 958, 220 U.S.P.Q. 592, 598 (Fed. Cir. 1983), *cert. denied,* 469 U.S. 835 (1984) ("When a properly claimed invention meets at least one stated objective, utility under 35 U.S.C. § 101 is clearly shown."). *See*, M.P.E.P. at 2100-29. Finding a lack of utility is also improper if a person of ordinary skill in the art would know of a use for the claimed invention at the time the application was filed. M.P.E.P. § 2107.01(II)(B) at 2100-[29-30].

Moreover, the burden is on the Examiner to establish why it is more likely than not that one of ordinary skill in the art would doubt *(i.e.,* "question") the truth of the statement of utility. M.P.E.P. § 2107.01(II)(A) at 2100-[31-32]. Thus, the Examiner must provide evidence sufficient to show that the statement of asserted utility would be considered "false" by a person of ordinary skill in the art. *Id.* The Examiner must also present countervailing facts and reasoning sufficient to establish that a person of ordinary skill would not believe the applicants' assertion of utility. *See id.*; *see also, In re Brana,* 51 F.3d 1560, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995). For the reasons set forth below, the Examiner has not met the burden that is necessary to establish and maintain a rejection for lack of utility under 35 U.S.C. § 101.

Contrary to the Examiner's comments, Applicants have set forth in the specification statements that clearly and fully describe the function of Human Cytokine

Polypeptide of the present invention and explain why Applicants believe the invention is useful. For example, the specification, at page 3, lines 5-8, teaches that polypeptides of the present invention may stimulate cell proliferation and/or differentiation. Furthermore, the specification, at page 37, lines 7-13, teaches that polypeptides of the invention have uses, for example, in the detection of neoplasia. Moreover, the specification, at page 37, line 15 to page 38, line 13, teaches examples of methods by which polypeptides of the present invention may be used, for example, in the detection of neoplasia. Applicants assert that such characterizations of the invention are sufficient to constitute a showing of utility.

In further support for the proposed utility of the instant invention, Applicants submit the teachings of Graf et al. (GenBank Accession No. CAC05581. (2000)) as Exhibit A and Oelgeschläger et al. (Nature, 405: pp 757-763. (2000)) as Exhibit B. Both references were published in 2000.

Graf et al. teach that the human twisted gastrulation homologue is identical in amino acid composition to the Human Cytokine Polypeptide of the present invention. They further teach that the twisted gastrulation gene is conserved from *Drosophila melanogaster* to *Homo sapiens* and that in humans this gene maps to the holoprosencephaly locus.

Oelgeschläger et al. teach that "[t]he *twisted gastrulation* gene encodes a secreted protein that is specifically required for the differentiation of amnioserosa cells in *Drosophila*" and that it functions by direct interaction with Bone Morphogenetic Proteins (BMPs). These authors further demonstrate that the twisted gastrulation protein performs

similar functions in vertebrates and in *Drosophila* and such functions are achieved by similar mechanisms in vertebrates and *Drosophila*.

From this evidence, one skilled in the art would appreciate that the pending claims of the present invention are indeed supported by specific and substantial utilities, which are entirely credible in light of the state of knowledge in the art. Not every protein can bind to and regulate the function of BMPs and thereby influence cell differentiation and tissue specification, neither does every gene map to the locus of a severe developmental disorder such as holoprosencephaly. Therefore Applicants respectfully submit that asserted utilities of the present invention are specific, substantial and credible.

Other than the conclusory statements that the invention lacks utility, the Examiner has presented no arguments as to why this asserted utility is not credible. In arguing that Applicants' asserted utility is not credible, the Examiner must attack (a) the logic underlying the assertion as seriously flawed or (b) the facts upon which the assertion is based as inconsistent with the logic underlying the assertion. *See*, Revised Interim Utility Guidelines Training Materials, p. 5. In the instant rejection, the Examiner has set forth no arguments as to why Applicants' logic (that Human Cytokine Polypeptide of the present invention has the activity of modulating cell proliferation and/or differentiation) is flawed or that the facts upon which the logic is based on, are inconsistent with the underlying assertion. Thus, the Examiner has failed to make even a *prima facie* showing that Applicants' asserted utility is not credible.

Moreover, Applicants respectfully submit that the Human Cytokine Polypeptide of the invention (such as, for example, the polypeptide shown as SEQ ID NO:2), has an immediate and specific utility. Such polypeptide may be used to stimulate cell

proliferation and/or differentiation and therefore, polypeptides of the instant invention, or agonists or antagonists thereof, may be used to treat and/or prevent disorders of cell differentiation and/or proliferation. Such polypeptides of the invention may be produced using Human Cytokine polynucleotides. *See*, *e.g.*., specification, at page 31, line 6 through page 33, line 20. Such polypeptides may then be used to generate antibodies specific for Human Cytokine polypeptides. *See*, *e.g.*, specification, at page 38, line 15 through page 39, line 17. The specification as originally filed teaches that "mRNA for human cytokine polypeptide is abundant in most major tissues including heart, brain, lung, placenta, kidney, skeletal muscle, ovary, testis, prostate and smooth muscle ", *See*, specification, at page 56, lines 4-6. The specification as originally filed also teaches that "above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or purify the polypeptide of the present invention". *See*, specification, at page 39, lines 11-12. Thus, polypeptides of the invention are supported by an immediate utility that is both specific and substantial.

In addition to the biological activity of Human Cytokine Polypeptide, Applicants have contemplated and disclosed therapeutic applications of Human Cytokine Polypeptide, for example, treatment in mammals, preferably humans, of disregulation of cell proliferation and/or differentiation, consistent with the biological activity of Human Cytokine Polypeptide. *See*, specification, at page 3, lines 5-8.

Applicants submit that these asserted utilities for Human Cytokine Polypeptide are specific (not every protein modulates cell proliferation and/or differentiation) and substantial ("the general rule [is] that the treatments of specific diseases or conditions meet the criteria of 35 U.S.C. § 101." (Revised Interim Utility Guidelines Training

Materials, p. 6)). In addition, Applicants submit that these utilities are credible and provide evidentiary support for this credibility in the form of Exhibits A and B. The Examiner has failed, however, to provide any countervailing statements as to why these particular utilities are not specific, substantial and credible.

In regard to these asserted therapeutic activities, Applicants note that there is no need to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty or provide actual evidence of success in treating humans where such a utility is asserted. M.P.E.P. § 2107.02 (I) at 2100-[33-34]. All that is required of Applicants is that there be a reasonable correlation between the biological activity and the asserted utility. *See*, *Nelson v. Bowler*, 626 F.2d 853, 857 (C.C.P.A. 1980). Moreover, "[u]sefulness in patent law, and in particular in the context of pharmaceutical inventions, *necessarily* includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans." *In re Brana, 51* F.3d *1560, 1568* (Fed. Cir. 1995) (emphasis added).

Even assuming, *arguendo,* the Examiner has established a *prima facie* showing that the claimed invention lacks utility, Applicants respectfully submit that they have rebutted the Examiner's showing by proffering sufficient evidence to lead one skilled in the art to conclude that the asserted utilities are more likely than not true. Applicants have directed the Examiner to the specification where clear and specific assertions are made of Human Cytokine polypeptide biological and therapeutic activity.

In view of the above, Applicants submit that the asserted utilities of the invention meet the statutory requirement set forth in 35 U.S.C. § 101. The Examiner has failed to

establish and maintain grounds as to why a rejection for lack of utility is proper. Accordingly, Applicants respectfully request that the rejection of claims 25-79 under 35 U.S.C. § 101 be withdrawn.

The Examiner has rejected claims 25-79 under 35 U.S.C. § 112, first paragraph, "since the claimed invention is not supported by either a specific and substantial asserted utility or a well-established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention".

Applicants respectfully disagree and traverse this rejection.

Applicants submit that the asserted utilities of the invention meet the statutory requirement set forth in 35 U.S.C. § 101 and that armed with the specification of the instant invention, one skilled in the art clearly would know how to use the claimed invention. Accordingly, Applicants respectfully request that the rejection of claims 25-79 under 35 U.S.C. § 112, first paragraph, be withdrawn.

II. Rejections under 35 U.S.C. § 112, first paragraph

The Examiner has rejected claims 34-42 under 35 U.S.C. § 112, first paragraph, as "containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention". In the interest of facilitating prosecution, Applicants submit herewith a declaration by Applicants' Agent regarding public availability of ATCC Deposit No. 97486 which contains a cDNA clone of the present invention.

Applicants submit that the Declaration submitted herewith fully addresses and satisfies the Examiner's concerns. Accordingly, Applicants respectfully request that the rejection of claims 34-42 under 35 U.S.C. § 112, first paragraph, be withdrawn.

Respectfully submitted,

Date: March 2, 2001

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MMW/JJK/BM/llc

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: SU, *et al.*

Application Serial No.: 09/416,267 Art Unit: 1646

Filed: October 12, 1999 Examiner: Mertz, P.

For: Human Cytokine Polypeptide Attorney Docket No.: **PF270P1**

Version with Markings to Show Changes Made

In the specification:

At Page 3, the paragraph beginning line 5 has been amended as follows:

It also is an object of the invention to provide human cytokine polypeptides, particularly human cytokine polypeptide, that ~~stimulates~~ stimulate cell proliferation and/or differentiation and may be employed to treat and/or prevent restenosis and inflammation.

At Page 33, the paragraph beginning line 14 has been amended as follows:

~~human~~ Human cytokine polypeptide polynucleotides and polypeptides may be used in accordance with the present invention for a variety of applications, particularly those that make use of the chemical and biological properties of human cytokine polypeptide. Among these are applications in cell proliferation and differentiation. Additional applications relate to diagnosis and to treatment of disorders of cells, tissues and organisms. These aspects of the invention are illustrated further by the following discussion.

At Page 37, the paragraph beginning line 7 has been amended as follows:

The present invention also relates to ~~a~~ diagnostic assays such as quantitative and diagnostic assays for detecting levels of human cytokine polypeptide protein in cells and tissues, including determination of normal and abnormal levels. Thus, for instance, a diagnostic assay in accordance with the invention for detecting overexpression of human cytokine polypeptide protein compared to normal control tissue samples may be used to detect the presence of neoplasia, for example. Assay techniques that can be used to determine levels of a protein, such as human cytokine polypeptide protein of the present invention, in a sample derived from a host are well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis and ELISA assays. Among these ELISAs frequently are preferred. An ELISA assay initially comprises preparing an antibody specific to human cytokine polypeptide, preferably a monoclonal antibody. In addition a reporter antibody generally is prepared which binds to the monoclonal antibody. ~~The~~ To the reporter antibody is attached a detectable reagent such as a radioactive, fluorescent or enzymatic reagent, in this example horseradish peroxidase enzyme.

In the claims:

Claims 17-19, 23, and 24 have been cancelled without prejudice.

1: CAC05581 **twisted gastrulation protein [Homo sapiens]**

BLink, Related Sequences, Nucleotide, Taxonomy, LinkOut

```
LOCUS       CAC05581      223 aa                      PRI       29-AUG-2000
DEFINITION  twisted gastrulation protein [Homo sapiens].
ACCESSION   CAC05581
PID         g9955693
VERSION     CAC05581.1  GI:9955693
DBSOURCE    embl locus HSA297391, accession AJ297391.1
KEYWORDS    .
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 223)
  AUTHORS   Graf,D., Timmons,P.M., Episkopou,V., Hitchin,M., Moore,G.,
            Fujiyama,A., Ito,T., Fisher,A.G. and Merkenschlager,M.
  TITLE     The twisted gastrulation gene is conserved from Drosophila to man
            and maps to the holoprosencephaly locus on 18p11.3
  JOURNAL   Unpublished
REFERENCE   2  (residues 1 to 223)
  AUTHORS   Graf,D.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-JUN-2000) Graf D., Lymphocyte Development Group, MRC
            Clinical Sciences Centre, Imperial College School of Medicine,
            Hammersmith Hospital, Du Cane Road, London, W12 ONN, UNITED KINGDOM
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     mat_peptide     26..223
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                     /coded_by="AJ297391.1:143..814"
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      121 fpvaeelshh enlvsfletv nqphhqnvsv psnnvhapys sdkehmctvv yfddcmsihq
      181 ckiscesmga skyrwfhnac cecigpecid ygsktvkcmn cmf
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The evolutionarily conserved BMP-binding protein Twisted gastrulation promotes BMP signalling

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Dorsal–ventral patterning in vertebrate and *Drosophila* embryos requires a conserved system of extracellular proteins to generate a positional information gradient. The components involved include bone morphogenetic proteins (BMP/Dpp), a BMP antagonist (Chordin/Short gastrulation; Chd/Sog) and a secreted metalloproteinase (Xolloid/Tolloid) that cleaves Chd/Sog. Here we describe *Xenopus* Twisted gastrulation (xTsg), another member of this signalling pathway. xTsg is expressed ventrally as part of the BMP-4 synexpression group and encodes a secreted BMP-binding protein that is a BMP signalling agonist. The data suggest a molecular mechanism by which xTsg dislodges latent BMPs bound to Chordin BMP-binding fragments generated by Xolloid cleavage, providing a permissive signal that allows high BMP signalling in the embryo. *Drosophila* Tsg also binds BMPs and is expressed dorsally, supporting the proposal that the dorsal–ventral axis was inverted in the course of animal evolution.

Dorsal–ventral patterning in vertebrates is regulated by a gradient of BMP activity. BMPs are expressed relatively uniformly in a wide area of the gastrulating *Xenopus* embryo and the gradient is thought to be generated by the localized secretion of BMP antagonists, such as Chordin and Noggin, by the dorsal lip or Spemann's organizer[1,2]. A further level of regulation is introduced by a secreted zinc metalloproteinase, Xolloid, which cleaves inactive Chordin–BMP complexes, resulting in the reactivation of BMP signalling in the embryo[3,4]. This model of dorsal–ventral patterning has been validated by genetic studies in zebrafish[5–10]. Chordin contains four cysteine-rich (CR) domains of about 70 amino acids each, and the Xolloid cleavage sites are located at conserved aspartic acid residues just downstream of CR1 and CR3 (refs 3, 11). Individual cysteine-rich domains, in particular CR1 and CR3, bind BMP, albeit with a 10-fold lower affinity than full-length Chordin[12]. Microinjection of CR1 or CR3 messenger RNA results in dorsalization and induction of secondary axes in *Xenopus* embryos. Thus, even after cleavage by Xolloid, the Chordin fragments can still inhibit BMP signalling[12]. This observation indicated that additional factors might be required to release BMP from the Chordin fragments generated by Xolloid to reactivate BMP signalling through its cognate receptor. In *Drosophila*, seven zygotic genes have been proposed to regulate dorsal–ventral patterning[1,13]. Among them, *decapentaplegic* (*dpp*) and *screw* (*scw*) encode BMP homologues that promote dorsal cell fates such as amnioserosa and inhibit development of the ventral central nervous system[13–16]. The *chordin* homologue *short gastrulation* (*sog*) is expressed ventrally and promotes central nervous system development[17–19].

The phenotype of *sog* loss-of-function mutants is intriguing: as expected for a Dpp/Scw antagonist, ventral structures are lost but, in addition, the amnioserosa is reduced. This result is paradoxical, as the amnioserosa is the dorsal-most tissue and therefore Sog, a BMP antagonist, is required for maximal BMP signalling[20–23]. A model proposed to explain the role of Sog in promoting peak Dpp activity suggests that Sog–BMP complexes may permit the diffusion of BMPs originating from more ventral regions, which are then released dorsally by the proteolytic activity of Tolloid[21]. The recent demonstration that BMPs remain bound to individual cysteine-rich domains, which remain intact in the Chordin proteolytic products[12], makes this interpretation unlikely, unless an additional factor that releases BMP from the cysteine-rich modules is proposed. The *twisted gastrulation* gene encodes a secreted protein that is specifically required for the differentiation of amnioserosa cells in *Drosophila*[24,25] and is a candidate for such a factor.

Here we show that the *Xenopus* homologue of *twisted gastrulation* (*xTsg*) shares sequence similarities with the cysteine-rich domains of Chordin, and is part of the BMP synexpression group[26]. Biochemical studies show that xTsg and *Drosophila* dTsg directly bind BMPs with dissociation constants in the low nanomolar range. In microinjection experiments, *xTsg* mRNA behaves as an agonist of BMP signalling, ventralizing the *Xenopus* embryo. xTsg competes efficiently with CR1 for binding to BMP and can bind full-length Chordin, forming a ternary complex containing Chordin, BMP and xTsg *in vitro*. The dorsalizing activity of CR1 is readily competed by wild-type xTsg, and is greatly potentiated by reducing endogenous xTsg activity. The results indicate that xTsg is involved in dorsal–ventral patterning, permitting peak BMP signalling by antagonizing the residual anti-BMP activity of the cleavage products of Chordin.

xTsg is expressed in ventral-most tissues

We isolated a full-length *xTsg* complementary DNA by using a human expressed sequence tag (EST) to probe a *Xenopus* gastrula library. The cDNA encodes a protein sharing 41% amino-acid identity with *Drosophila* Tsg (dTsg), 89% identity with the partial human *Tsg* sequence and 94% identity with a mouse EST. The xTsg sequence contains a signal peptide, as expected for a secreted protein, and two conserved domains containing multiple cysteines at its amino and carboxy termini (Fig. 1a). Whole-mount *in situ* hybridization and polymerase chain reaction with reverse transcription (RT–PCR) showed that abundant *xTsg* maternal transcripts are distributed throughout the animal half of the embryo during cleavage stages (Fig. 1b, and data not shown). At the late gastrula stage, maternal transcripts decrease and zygotic transcripts appear specifically in the ventral region of the embryo (Fig. 1c). After neurulation, *xTsg* transcripts surround ventrally the closed blastopore slit and the neural tube (Fig. 1d). At the tailbud stage, *xTsg* transcripts are detected in the postanal region, heart and dorsal eye (Fig. 1e, f) and closely mimic the expression patterns of *BMP-4* and *BAMBI*[27,28] (Fig. 1e–j). The postanal region of xTsg expression derives from the ventral-most tissue[29] of the gastrula embryo, as

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illustrated by the transplantation experiment shown in Fig. 1k and l. We conclude that *xTsg* is part of the *BMP-4* synexpression group[26] and is expressed in the ventral pole of the embryo.

xTsg has ventralizing activity

Microinjection of *xTsg* mRNA into each blastomere of the four-cell embryo resulted in the reduction of dorso-anterior structures (Fig. 2a). This phenotype was reminiscent of the *chordin* zebrafish mutant[5], and of *Xenopus* embryos microinjected with *Xolloid* mRNA[3,30] or with low doses of *BMP-4* mRNA[31]. Molecular marker analyses showed that dorsal ectoderm (*Sox-2*) and dorsal mesoderm (*MyoD* and *Shh*) are reduced and that ventral tissues (*BMP-4*) are expanded in *xTsg*-injected embryos (Fig. 2b–e). We conclude from these results that *xTsg* has ventralizing activity.

To determine whether xTsg functions in the BMP pathway, epistatic analyses were performed by injecting *xTsg* mRNA into a ventral blastomere together with a dominant-negative BMP receptor (*tBR*) or extracellular BMP antagonists (*noggin*, *chordin* and *CR1*). *xTsg* had no effect on the formation of secondary axes by tBR, indicating that it may ventralize the embryo upstream of the BMP receptor (Fig. 3b). To test whether xTsg could antagonize the dorsalizing activity of the proteolytic fragments of Chordin, we generated a construct containing the N-terminal CR1 domain and terminating at the Xolloid cleavage site[11]. *CR1* mRNA induced secondary axes (although at 32-fold higher molar concentrations than those required for full-length *chordin* mRNA, Fig. 3g), which were blocked by co-injection of *xTsg* (Fig. 3h). *Xolloid* mRNA was able to block the activity of full-length *chordin*, as shown previously[3], but had no effect on secondary axes induced by the *CR1* construct (Fig. 3f, i). This indicates that xTsg efficiently antagonizes high doses of CR1 downstream of Xolloid cleavage. The ability of xTsg to inhibit full-length *Chordin* mRNA (Fig. 3d, e) presumably results from the activity of uniformly expressed Xolloid proteases in the early embryo[30]. That the ventralizing activity of xTsg requires Xolloid cleavage is confirmed by the observation that a CR1 construct containing 80 additional amino acids downstream of the first Xolloid site could not be antagonized by co-injection of either *xTsg* or *Xolloid* mRNAs (unpublished observations). The ventralizing activity of *xTsg* appears to be specific for the Chordin/BMP pathway, as inhibition of BMP by *noggin* was not affected by co-injection of *xTsg* mRNA (Fig. 3c). These epistatic studies are consistent with a model in which *xTsg* would ventralize the embryo by antagonizing the residual anti-BMP activity of Chordin proteolytic cleavage products (Fig. 3j).

xTsg is a BMP-binding protein

When the N-terminal domain of xTsg was compared with the cysteine-rich domains of Chordin, we noticed sequence similarities (Fig. 4a). As cysteine-rich domains are BMP-binding modules[12], we tested whether epitope-tagged xTsg secreted by transfected 293T cells could bind BMP-4. xTsg did bind to BMP-4 in solution, and

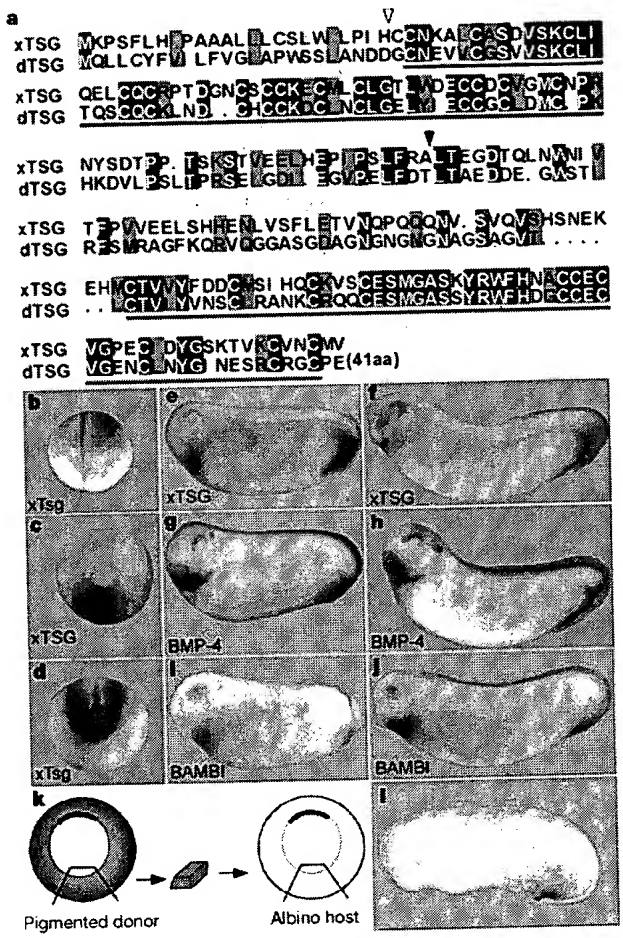

Figure 1 xTsg shares two conserved regions with dTsg and is co-expressed with BMP-4 and BAMBI. **a**, Alignment of xTsg and dTsg. The potential cleavage site for the signal peptide (open arrowhead), the conserved regions (red bars) and the position at which the N-terminal and C-terminal fragments were divided (black arrowhead) are indicated. **b–f**, Whole-mount *in situ* analysis of *xTsg* expression. **b**, Four-cell stage; **c**, late gastrula (posterior view); **d**, stage 14. **e**, **f**, At stages 20 and 24, *xTsg* expression marks the postanal region, the heart and the dorsal eye. **g**, **h**, BMP-4 and **i**, **j**, BAMBI expression at stages 20 and 24, respectively. **k**, Experimental design of an isotopic and isochronic transplant of the ventral-most tissue at early gastrula (stage $10\frac{1}{2}$) from a pigmented donor to an albino host ($n = 11$). **l**, The ventral pigmented transplanted tissue populates the perianal region of the early tailbud embryo.

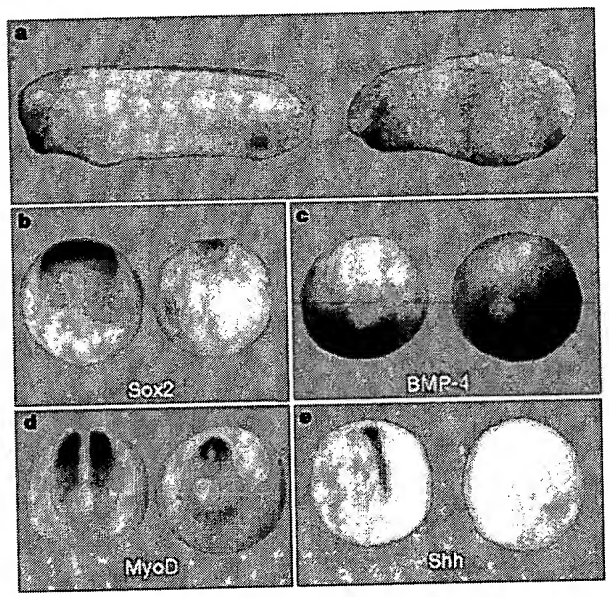

Figure 2 Microinjection of *xTsg* mRNA leads to ventralization of the embryo. **a**, Control embryo (left) and embryo with *xTsg* mRNA microinjected into each animal cell at the eight-cell stage (500 pg total; right), leading to the reduction of dorso-anterior structures. **b–e**, *In situ* analysis of late gastrulae microinjected with *xTsg* mRNA (500 pg per blastomere). Injected embryos (right) and uninjected controls (left) in dorsal view. **b**, *Sox2*; **c**, *BMP-4*; **d**, *MyoD*; **e**, *Shh*. The changes in gene expression are characteristic of ventralization in *Xenopus*[3].

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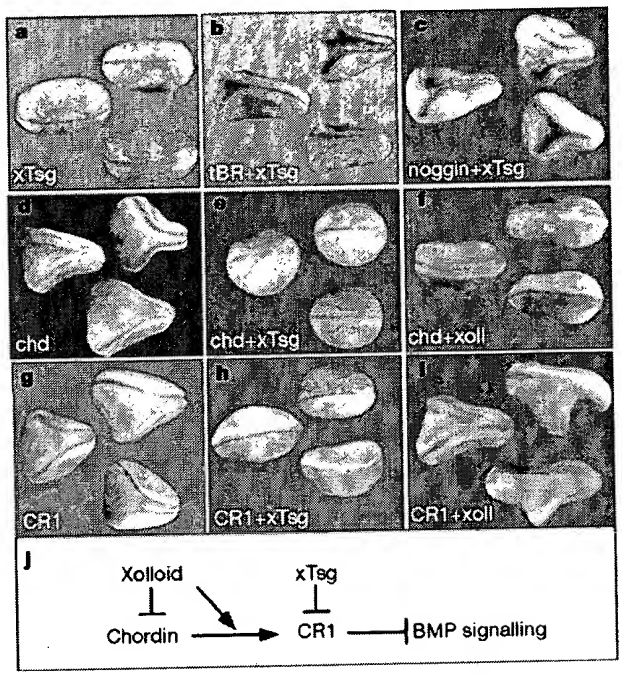

Figure 3 xTsg blocks secondary axis formation by CR1 downstream of Chordin cleavage by Xolloid. **a**, Microinjection of 250 pg *xTsg* mRNA. **b**,**c**, The induction of secondary axes by 200 pg *tBR* (dominant-negative BMP receptor; **b**) or 5 pg *noggin* mRNA (**c**) is not affected by co-injection of 250 pg *xTsg* mRNA. **d**, Injection of 10 pg *chordin* mRNA induces the formation of secondary axes that are inhibited by co-injection of 250 pg *xTsg* mRNA (**e**) or 200 pg *Xolloid* mRNA (**f**). **g**, Secondary axes induced by injections of 80 pg *CR1* mRNA (a 32-fold molar excess compared with full-length *chordin* mRNA) are inhibited by co-injection of 250 pg *xTsg* mRNA (**h**) but not by co-injection of 200 pg *Xolloid* mRNA (**i**). Similar results were obtained in at least three independent experiments, with $n = 22–52$ embryos per mRNA combination. All embryos were injected once ventrally at the eight-cell stage. **j**, Summary of the epistatic studies. Xolloid inhibits Chordin activity but not that of CR1, and xTsg can block CR1 function. These epistatic analyses place the xTsg ventralizing activity downstream of Xolloid cleavage and upstream of the receptor.

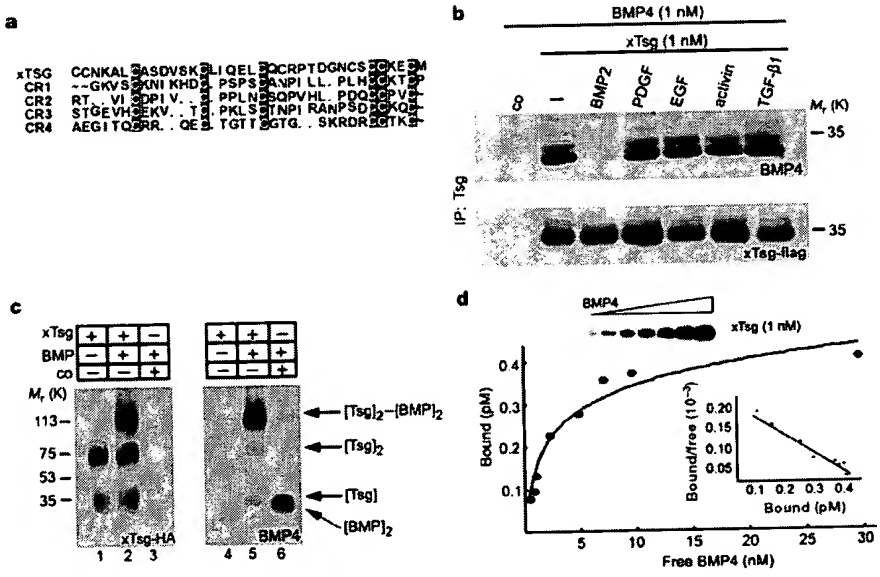

Figure 4 xTsg binds to BMP specifically and directly. **a**, Sequence similarities between Chordin cysteine-rich modules and the N-terminal domain of xTsg. **b**, Western blot analyses of BMP-4 (1.0 nM) bound to xTsg (1.0 nM) after immunoprecipitation by xTsg in the presence or absence of a 10-fold excess of various growth factors. The control (co) lanes in this and Figs 5–7 contain conditioned medium from mock-transfected 293T cells. To measure the recovery of xTsg after immunoprecipitation, the same membrane was stripped and probed for xTsg protein (bottom). **c**, Crosslinking analysis of xTsg–BMP-4 complexes with DSS. Left, immunoblot probed for xTsg. Right, the same membrane probed for BMP-4 after stripping. **d**, Equilibrium binding of increasing concentrations of BMP-4 (0.2–30 nM) to 1 nM Tsg. Binding was for 3 h at 4 °C, and bound and free BMP-4 were separated by immunoprecipitation; crosslinking agents were not used. The amount of bound BMP-4 was determined as described[12]. Each data point was in duplicate, and two independent experiments were performed for each protein. Scatchard analyses of the dTsg data gave an apparent K_d of 2.5 nM. The same results and K_d were obtained for the xTsg protein as illustrated by the anti-BMP-4 immunoblot (inset and data not shown).

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this interaction was specific as it could be competed by BMP-2 but not by a 10-fold excess of platelet-derived growth factor, epidermal growth factor, Activin or transforming growth factor (TGF)-β1 (Fig. 4b). We used chemical crosslinking with disuccinimidyl suberate (DSS) to determine whether this molecular interaction was direct. After separation of the complexes under reducing conditions, haemagglutinin (HA)-tagged xTsg (1 nM) formed mostly dimers and a small amount of monomers (Fig. 4c, lane 1). In the presence of 1 nM BMP-4, the xTsg dimers shifted to a molecular mass consistent with the binding of one BMP-4 dimer (Fig. 4c, lanes 2 and 5). Using an immunoprecipitation assay, the apparent dissociation constant for equilibrium binding of BMP-4 to xTsg (K_d) was determined by Scatchard analysis and found to be about 2.5 nM for both *Xenopus* and *Drosophila* Tsg (Fig. 4d and data not shown). To map the BMP binding domain, we generated constructs consisting of the conserved domains of xTsg and dTsg separated at the sites indicated in Fig. 1a and prepared secreted proteins. As shown in Fig. 5a and b, the BMP-binding activity resided in the N-terminal region of xTsg and dTsg, that is, in the domain that shares sequence similarities with the BMP-binding modules of Chordin. We conclude that xTsg is a secreted BMP-binding protein that functions in embryos as an agonist of BMP signalling. The other secreted BMP-binding proteins identified, such as Chordin, Noggin, Follistatin and members of the Cerberus family, are antagonists of BMP activity[2,32–34].

xTsg competes with CR-1 to bind BMP

We next tested whether Chordin and xTsg could compete for BMP binding. Full-length Chordin (1 nM) was immunoprecipitated in the presence of 0.5 nM BMP-4 and increasing amounts of xTsg protein. Instead of competing for the limited amounts of BMP-4, xTsg stimulated binding of BMP-4 to full-length Chordin (Fig. 5c, lanes 2–5). Further analyses showed that xTsg itself could bind to Chordin even in the absence of added BMP (Fig. 5d, lane 2). This binding was confirmed using a cell-culture assay in which permeabilized cells transfected with *xTsg* cDNA were stained with a Chordin–alkaline phosphatase fusion protein[35,36] (Fig. 5e, f). The binding of xTsg to Chordin required intact xTsg, as neither the N- nor the C-terminal fragments sufficed for the interaction (Fig. 5d, lanes 3 and 4). The observation that the N terminus is unable to bind Chordin but can still bind BMP-4 suggests that these two interactions occur through different sites.

Crosslinking experiments with DSS showed that xTsg stimulates the binding of BMP-4 to full-length Chordin by forming a trimolecular complex with a relative molecular mass of about 220,000 (M_r 220K) (Fig. 6a, lane 3). This shift is consistent with the crosslinking of one dimer of BMP-4 and one dimer of xTsg per Chordin monomer, as depicted in Fig. 6b. To investigate the molecular mechanism by which xTsg can antagonize the activity of CR1, equimolar amounts (1 nM) of BMP-4, CR1 and xTsg were incubated for 1 h at room temperature before crosslinking with DSS. When CR1 and BMP-4 were incubated together, a complex of M_r 50K was formed, corresponding to the binding of a CR1 monomer to a BMP dimer (Fig. 6a, compare lanes 1 and 4). Incubation of xTsg

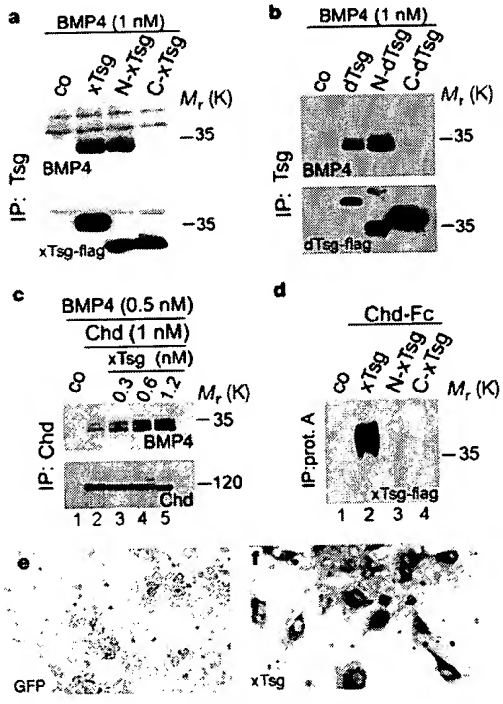

Figure 5 The Tsg N-terminal domain is sufficient to interact with BMP-4, but not with Chordin. **a**,**b**, Western blot analyses of BMP-4 (1 nM) bound to 1 nM of *Xenopus* or *Drosophila* Tsg. As a loading control, the same membranes were stripped and probed against Tsg (bottom). **c**, Western blot analyses of BMP-4 (0.5 nM) bound by 1 nM Chordin (lane 2) in the presence of 0.3, 0.6 and 1.2 nM xTsg (lanes 3, 4 and 5, respectively). The presence of xTsg increases the binding of BMP-4 to Chordin about fourfold (quantification by Phosphoimager, not shown). Bottom, same membrane probed with anti-Chordin antibody. **d**, Immunoprecipitation with protein A-sepharose of a mixture of 1 nM Chordin–Fc fusion protein with 1 nM xTsg, N-xTsg and C-xTsg. **e**,**f**, The Chordin–xTsg interaction was confirmed by binding to COS cells transfected with green fluorescent protein (negative control) or with xTsg cDNA, which were permeabilized and stained with a Chordin–alkaline phosphatase fusion protein.

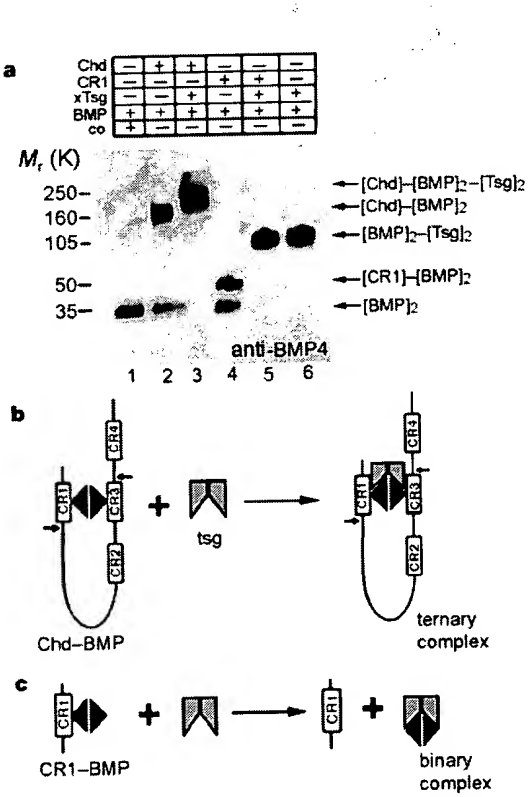

Figure 6 xTsg competes for binding of BMP-4 with CR1 but not with full-length Chordin. Crosslinking analyses were performed using DSS. **a**, Lane 1, BMP-4 (1 nM). 1 nM Chd and 1 nM BMP-4 form a complex of about 150K (lane 2) that shifts to ~220K in the presence of 1 nM xTsg (lane 3). When 1 nM CR1 was crosslinked to BMP-4 a 50K complex was detected (lane 4). When xTsg was added a complex of around 100K was produced exclusively (lane 5), which was identical to the [BMP]$_2$–[xTsg]$_2$ complex (lane 6). **b**, **c**, Diagrams summarizing the crosslinking results. The small arrows in Chordin indicate the Xolloid cleavage sites.

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and BMP-4 resulted in the formation of a complex of about 100K, corresponding to a dimer of xTsg bound to a dimer of BMP-4 (Fig. 6a, lane 6). Remarkably, when CR1, BMP-4 and xTsg were incubated together, only the $[xTsg]_2$–$[BMP]_2$ complex was formed (Fig. 6a, lane 5). Furthermore, when BMP-4 and CR1 were preincubated for 1 h, BMP-4 could still be dislodged from these preformed complexes by addition of xTsg (not shown). We conclude from these results that, in contrast to full-length Chordin, CR1 bound to BMP-4 is released in the presence of xTsg, resulting in a binary complex of BMP-4 and xTsg (Fig. 6c).

Endogenous xTsg antagonizes CR1 activity

To investigate the effect of loss of function of endogenous xTsg, we used two complementary approaches. A construct secreting the C-terminal conserved domain (*dn-xTsg*) was found to have dominant-negative effects (see below) and double-stranded xTsg RNA (RNAi) was also found to interfere with endogenous *xTsg* function. RNAi potently and specifically inhibits gene expression in a number of species[37–39]. *xTsg* RNAi (100 pg) was injected into each animal blastomere at the eight-cell stage together with epitope-tagged *xTsg* and *CR1* mRNAs. As shown in the inset of Fig. 7h, RNAi inhibited the expression of secreted xTsg but not of CR1 protein, indicating that RNAi is effective and specific in *Xenopus*. Microinjection of *dn-xTsg* or of *RNAi* resulted in abnormal development of the postanal region, in particular loss of the ventral fin and shortening of the tail which were only evident at swimming tadpole stages (data not shown). At earlier stages of development, only mild defects in the perianal region were observed (Fig. 7d, e); these minimal early phenotypes may be due to the strong maternal contribution of *xTsg* (Fig. 1b). However, both *dn-xTsg* mRNA and RNAi greatly potentiated the dorsalizing effects of low doses of *CR1* mRNA injected into all blastomeres of four-cell embryos (compare Fig. 7a, f and h). These effects could be partially rescued by co-injection of wild-type *xTsg* mRNA, although the rescue was more effective for *dn-xTsg* (Fig. 7f, g) than for *xTsg* RNAi (Fig. 7h, i).

The results could be confirmed using molecular markers in ectodermal explants. As can be seen in Fig. 7j, *dn-xTsg* and *xTsg* RNAi potentiated the induction of the neural marker *NCAM*, as well as the inhibition of the epidermal marker *cytokeratin*, by *CR1* mRNA (lanes 6, 8 and 10). The neuralizing effect of *CR1*, but not that of *tBR*, could be reversed by *xTsg* mRNA (lanes 6 and 7, and data not shown). The strong neuralizing effects observed in the presence of *dn-xTsg* or RNAi could also be reversed by full-length *xTsg* mRNA (lanes 9 and 11). These loss-of-function experiments indicate that endogenous levels of xTsg activity are sufficient to inhibit the dorsalizing effects of microinjected CR1.

Discussion

The *Xenopus* homologue of *dTsg* is a member of the BMP-4 synexpression group[26], and is transcribed in the ventral-most region of the embryo. Overexpression of this secreted protein ventralizes the embryo through a molecular mechanism functioning upstream of the BMP receptor (Fig. 3b). Epistasis experiments support a model in which xTsg would promote BMP signalling downstream of Chordin cleavage by the metalloproteinase Xolloid. Chordin is abundantly secreted by the dorsal pole of the embryo, reaching concentrations of 6–12 nM in the extracellular space of Spemann's organizer[40]. Chordin binds BMPs through cysteine-rich modules[12]. Xolloid cleaves Chordin downstream of the two cysteine-rich domains that have the highest affinity for BMP binding[3,11,12]. These proteolytic digestion products retain residual BMP-binding activity and can still inhibit the interaction between BMP and its cognate receptor[12]. Our results show that xTsg binds BMP directly in the low nanomolar range and will compete effectively with a cysteine-rich module for binding to BMP, leading to the formation of xTsg–BMP complexes that permit BMP signalling (Figs 2, 3 and 6). In this way, xTsg would promote BMP activity by dislodging the growth factor from an inhibitor in the extracellular space. Interfering with endogenous xTsg by two independent approaches, *dn-xTsg* and RNAi, greatly increased the ability of CR1 to inhibit BMP. *xTsg* mRNA did not cooperate with *BMP-4* mRNA in the induction of the target gene *Xvent-1* (ref. 26) in animal cap explants, nor did xTsg facilitate the binding of BMP-4 to recombinant BMP receptor IA (ref. 12) in biochemical assays (data not shown). This indicates that in the absence of Chordin cleavage

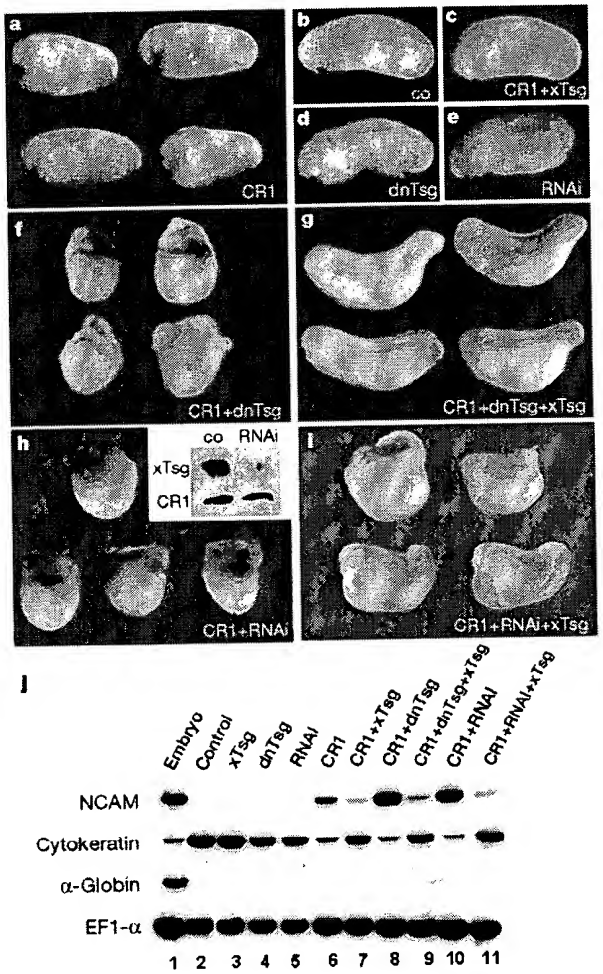

Figure 7 Loss of function of endogenous xTsg potentiates the activity of CR1 mRNA. Four-cell embryos were injected into each cell in the marginal region and cultured to stage 25. **a**, Injection of CR1 results in slight dorsalization compared with control uninjected embryos (**b**). **c**, Co-injection of *xTsg* and *CR1* mRNA prevents dorsalization by *CR1*. Injection of *dn-xTsg* (**d**) or RNAi (**e**) disrupts the development of the perianal region leading to shortening of the tail at later stages (not shown); at these early tadpole stages the phenotype was minimal. Co-injection of *CR1* and *dn-xTsg* mRNA (**f**) or RNAi (**h**) leads to strong synergistic dorsalization of the embryos, which can be partially rescued by co-injecting full-length *xTsg* mRNA (**g**, **i**). **h**, Inset, co-injection of HA-tagged *xTsg* mRNA and Myc-tagged *CR1* mRNA with or without xTsg RNAi. Ectodermal explants were dissociated[3], incubated for 14 h at 16 °C, and secreted proteins analysed by western blot. **j**, RT–PCR analysis of stage 25 ectodermal explants of embryos injected at the eight-cell stage. NCAM induction reflects inhibition of BMP signalling by *CR1* (lane 6), which is potentiated by co-injection of *dn-xTsg* or RNAi (lanes 8 and 10). The induction of NCAM by CR1, as well as its potentiation by co-injection of *dn-xTsg* or RNAi, was inhibited by co-injection of wild-type *xTsg* (lanes 7, 9, 11). The epidermal marker gene cytokeratin is regulated reciprocally by the injected RNAs and serves as a reporter of endogenous BMP signals. α-Globin was used as a mesodermal marker and EF1-α as loading control. The amounts of mRNA per blastomere injected were *CR1*, 20 pg; *xTsg*, 200 pg; *dn-xTsg*, 100 pg; and RNAi, 100 pg.

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products xTsg does not increase BMP signalling. Unlike CR1, the binding of full-length Chordin to BMP-4 is enhanced by xTsg, leading to the formation of a ternary complex that can be cross-linked *in vitro*; perhaps xTsg results in more efficient cleavage of Chordin by Xolloid.

The initial impetus to investigate whether xTsg bound BMP was provided by the observation that the N-terminal conserved domain shared amino-acid similarities with Chordin cysteine-rich repeats. This similarity was found to be functionally significant, as this domain contains the BMP-binding activity of xTsg. Cysteine-rich modules of the Chordin type are found in many extracellular proteins such as von Willebrand factor, thrombospondin, Nel and fibrillar procollagens. The cysteine-rich domain in procollagen IIA binds BMP and TGF-β1 (refs 12, 41) and is responsible for the dorsalizing activity of procollagen IIA in *Xenopus*[12]. It is therefore possible that the molecular mechanism described here for xTsg may provide a more general paradigm for signalling in the extracellular space. Similar functions in the release of latent growth factors bound to cysteine-rich domains could be executed by other secreted proteins that share structural similarities with the N-terminal repeat of xTsg, such as members of the connective tissue growth factor (CTGF) and insulin-like growth factor-binding protein (IGFBP) families[24,42]. In addition, the Cripto and Oep (one-eyed pinhead) proteins, which provide a permissive function for signalling by nodal TGF-β family members in mouse and zebrafish[43,44], share sequence similarities to xTsg in their conserved EGF-like domains (data not shown).

In *Drosophila*, loss of function of *dTsg* results in the loss of amnioserosa, which requires maximal amounts of Dpp/Scw signalling[14–16], but does not affect the rest of the dorsal–ventral pattern[24]. Overexpression of *dTsg* driven by a promoter expressed in the ventral-most region of the embryo rescues dorsal amnioserosa formation in a *dTsg* mutant background, indicating that the protein can diffuse throughout the embryo[25]. As *dTsg* does not affect other dorsal–ventral fates in *Drosophila*, it was thought to provide a permissive signal required for amnioserosa differentiation after cells have been exposed to peak levels of Dpp/Scw signalling[24,25]. Our results show that *Drosophila* Tsg can bind BMPs through its N-terminal domain (Fig. 5b). *Drosophila* Sog, although not yet demonstrated to bind Dpp/Scw/BMP in biochemical studies, is cleaved by Tolloid at three sites in the presence of BMP[45]. Two of these cleavage sites are located at similar positions to those in *Xenopus* Chordin[3]. We propose that dTsg can act only in the amnioserosa because its function is to release active Dpp/Scw from the cleavage fragments of Sog by Tolloid. Peak signalling would be mediated by Dpp/Scw originating in more ventral regions and reaching the dorsal region by diffusion as a complex with Sog[21]. Release would occur only in dorsal-most regions in which maximal levels of dTsg, Tolloid, and fragments of Sog bound to BMP are prevalent. In more ventral regions, full-length free Sog would be in excess[21] and would inhibit any released BMPs. This would be particularly true in the presence of dTsg in dorso-lateral regions, in view of the observation that xTsg increases the binding of BMP to full-length Chordin (Fig. 5c). This model has the attraction of explaining the paradoxical effects of Sog, which inhibits Dpp/Scw signalling in ventral ectoderm but promotes it in the most dorsal regions of the embryo[20,46]. By introducing dTsg as an additional factor that permits the release of Dpp/Scw from inactive complexes in the amnioserosa, it is not necessary to invoke positive (that is, non-inhibitory) signalling effects of Sog proteolytic cleavage products to account for the Tolloid-dependent long-range effects of Sog diffusion on peak Dpp signalling[21–23].

One of the great surprises of developmental genetics has been the evolutionary conservation of molecular mechanisms. The best known example is the discovery that conserved sets of Hox genes determine the anterior–posterior axis in all bilateral animals[47,48]. The activities and expression patterns of Dpp/BMP-4, Sog/Chordin and Tolloid/Xolloid have led to the view that the dorsal–ventral axis has been inverted in the course of evolution[1,18]. We now show that *Drosophila Tsg*, a gene expressed in the dorsal-most blastoderm, has a vertebrate homologue expressed in the ventral-most tissues of the *Xenopus* embryo. *xTsg* is part of an intricate extracellular signalling pathway in which the two poles of the dorsal–ventral pattern are defined by sources of secreted Sog/Chordin and dTsg/xTsg in opposite sides of the embryo. The results are consistent with the idea that the ventral side of the arthropod is homologous to the dorsal side of the vertebrate, as proposed by Geoffroy Saint-Hilaire[49].

Note added in proof: As this work was being reviewed, it was reported[50] that alternative Sog processing may generate protein forms with increased BMP inhibitory activity. In the light of our data, some of those findings might be explained by the formation of trimolecular complexes consisting of Tsg, Dpp and Sog fragments that are insensitive to Tolloid cleavage. □

Methods

DNA constructs

We screened a *Xenopus* gastrula cDNA library with the coding sequence of the *hTsg* EST; a full-length clone was isolated, sequenced and subcloned in the expression vector pCS2[1]. *xTsg* and *dTsg* were epitope-tagged by PCR either using a 3′ primer including an HA tag before the stop codon or by amplification of *xTsg* or *dTsg* subclones lacking the leader sequence and cloning into an expression vector including the chordin signal peptide and a Flag tag sequence (*pchd–Flag*, constructed by S. Piccolo). *Pchd–Flag* was used to generate *N-xTsg*, *C-xTsg*, *N-dTsg* and *C-dTsg*. The *Xenopus* pCS2–*CR1* construct was generated by PCR from pCS2–*chordin*[3] using a 5′ vector primer and a 3′ primer including Chordin sequences up to Asn 146, followed by a Myc tag.

Protein binding and crosslinking

Proteins were obtained by transient transfection of 293T cells (xTsg, dTsg) or S2 cells (Ch–dAP, Chd–Fc). Immunoprecipitations and equilibrium binding assays were performed as described[12]. For crosslinking[40], we incubated protein mixtures containing BMP-4 (R&D Systems) for 1 h at room temperature in 80 μl of PBS; disuccinimidyl suberate (Pierce) was then added to a final concentration of 0.85 mM and incubated at room temperature for a further 30 min. The reaction was stopped by adding 4 μl 1 M Tris-HCl (pH 7.4) and samples were electrophoresed in SDS gels under reducing conditions. For secretion-trap cell staining[35], COS-7 cells were transiently transfected and after two days fixed (1.8% paraformaldehyde for 15 min), permeabilized (0.1% TritonX-100 for 15 min), and incubated for 1 h at room temperature with Chordin–alkaline phosphatase fusion protein. Embryo manipulations and RNA analyses were as described[12].

Received 20 March; accepted 23 May 2000.

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Acknowledgements

We thank J. Fessler, L. Zipursky and members of our laboratory for comments on the manuscript, and U. Tran and A. Cuellar for technical assistance. M.O. and J.L. are HFSPO and Pew postdoctoral fellows, respectively. This work was supported by the NIH and the Howard Hughes Medical Institute.

Correspondence and requests for materials should be addressed to E.M.D.R. (e-mail: derobert@hhmi.ucla.edu). The accession number for *xTsg* is AF245221. Human and mouse *Tsg* homologues are encoded by ESTs AA486291 and AW258143.

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Twisted gastrulation is a conserved extracellular BMP antagonist

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Bone morphogenetic protein (BMP) signalling regulates embryonic dorsal–ventral cell fate decisions in flies, frogs and fish[1]. BMP activity is controlled by several secreted factors including the antagonists chordin and short gastrulation (SOG)[2,3]. Here we show that a second secreted protein, Twisted gastrulation (Tsg)[4], enhances the antagonistic activity of Sog/chordin. In *Drosophila*, visualization of BMP signalling using anti-phospho-Smad staining[5] shows that the *tsg* and *sog* loss-of-function phenotypes are very similar. In S2 cells and imaginal discs, TSG and SOG together make a more effective inhibitor of BMP signalling than either of them alone. Blocking Tsg function in zebrafish with morpholino oligonucleotides causes ventralization similar to that produced by chordin mutants. Co-injection of sub-inhibitory levels of morpholines directed against both Tsg and chordin synergistically enhances the penetrance of the ventralized phenotype. We show that Tsgs from different species are functionally equivalent, and conclude that Tsg is a conserved protein that functions with SOG/chordin to antagonize BMP signalling.

TSG is required to specify the dorsal-most structures in the *Drosophila* embryo, for example amnioserosa[4]. Mutations in the BMP-like ligands, Decapentaplegic (DPP) and Screw (SCW), the BMP inhibitory factor SOG, or the SOG-processing enzyme Tolloid (TLD), also cause loss of the amnioserosa, even though some of these products seem to have opposing biochemical functions[2,6–8]. To place TSG activity relative to the biochemical function of these other factors, we examined its loss-of-function phenotype using molecular markers (Fig. 1A). The phenotype of *tsg* mutants (Fig. 1A, c, g, l) is most similar to that produced by loss of the BMP antagonist SOG (Fig. 1A, b, f, k) rather than that produced by loss of the ligands DPP or SCW (data not shown), or the SOG-processing protease TLD (Fig. 1A, d, h, m). In both *tsg* and *sog* mutants, the dorsal marker Rhomboid (*rho*) expands (Fig. 1A, b, c)[9], whereas in *tld* mutants no *rho* expression is observed (Fig. 1A, d)[8]. In contrast, mutations in *tsg*, *sog* and *tld* eliminate expression of other presumptive amnioserosa markers including Race (Fig. 1A, g, f, h), Hindsight (data not shown) and Zerknüllt (*zen*) (data not shown). To determine whether the response of these is indicative of different threshold levels of DPP signalling[10], we used an anti-phospho-Smad antibody[5] to directly visualize the levels of ligand signalling. Wild-type embryos accumulate phosphorylated mother against DPP (P-MAD) in an 18–20-cell-wide dorsal stripe at mid-cellulariza-

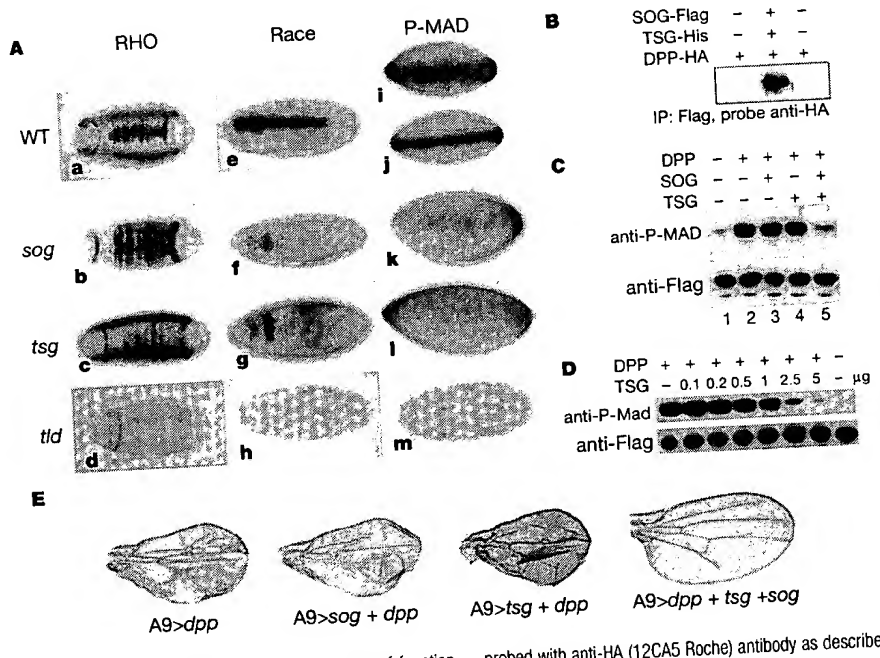

Figure 1 Tsg and SOG synergistically inhibit DPP signalling. **A**, The *tsg* loss-of-function phenotype is similar to that of *sog*. **a**–**h**, *In situ* hybridization of a Rhomboid RNA probe and a Race RNA probe to wild-type (WT), sog^{YS06}, tsg^{XB86} and tld^{B4} mutant embryos. Mutant embryos were identified by the absence of *lacZ* expression supplied by a marked balancer chromosome. All embryos are at the mid-cellular-blastoderm or early gastrulation stage; anterior is to the left and the view is dorsal. **i**–**m**, Anti-phospho-MAD staining of WT (**i**, **j**), sog^{YS06} (**k**), tsg^{XB86} (**l**) and tld^{B4} (**m**) mutant embryos. The embryos in **i** and **j** are dorsal side up, and in **k**–**m** they are viewed laterally. **B**, TSG and SOG form a high-affinity complex with DPP. S2 cells were transfected with DPP-HA and either SOG alone or SOG and TSG. After induction with Cu^{+2}, the supernatants were harvested and immunoprecipitated (IP) with anti-Flag antibody, transferred to a PVDF membrane and probed with anti-HA (12CA5 Roche) antibody as described[9]. **C**, TSG and SOG synergistically inhibit DPP signalling in S2 cells. In lanes 2–5, 20 ng purified DPP was added. Lane 1 was mock treated with buffer. In lanes 3–5, 1.25 μg SOG, 1 μg TSG or 1 μg of each were premixed with DPP and added to cells. The top panel was probed with anti-P-MAD antibody; the bottom with anti-Flag antibody. **D**, TSG alone inhibits DPP signalling in S2 cells at high concentrations. Cells were transfected with MAD-Flag as above, and then treated with 20 ng DPP and the indicated levels of TSG. **E**, TSG and SOG together form an effective inhibitor of DPP *in vivo*. Transformant lines containing UAS *dpp*, *sog* and *tsg* constructs[9,30] were crossed in the combinations indicated to the GAL4-A9 driver[30].

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tion (Fig. 1A, i) that rapidly resolves into an 8–9-cell-wide stripe (Fig. 1A, j) of more intensely stained cells just as gastrulation starts. Although an underlying gradient of activity not detectable by this method may exist[7,11,12] these results instead suggest that DPP/SCW activity is distributed in a sharp on–off pattern that resolves into a narrow stripe of dorsal cells, which—posterior to the cephalic furrow—corresponds in width to those cells labelled by the amnioserosa markers Race and Hindsight. In *sog* and *tsg* mutants (Fig. 1A, k, l), P-MAD fails to refine and intensify, whereas in *tld* mutants (Fig. 1A, m) P-MAD activity is below the level of detection in all dorsal cells. We suggest that the low, uniform levels of P-MAD seen in *sog* and *tsg* mutants are sufficient to activate *rho*, but not *race*, *hnd* or *zen* transcription.

As the phenotypes of *tsg* and *sog* mutants are similar, we sought to determine whether TSG can enhance the binding of SOG to ligand. Co-immunoprecipitation of DPP by SOG is greatly enhanced when these two factors are coexpressed in S2 cells along with TSG (Fig. 1B). To test whether the combination of SOG and TSG blocks DPP signalling better than SOG alone, we developed an S2 cell-culture assay for DPP signalling (Fig. 1C). At high concentration TSG alone can block DPP signalling (Fig. 1D); however, at lower concentration, the combination of TSG and SOG together dramatically reduces the DPP-dependent accumulation of P-MAD much more efficiently than either could alone. *In vivo* overexpression of *sog* and *tsg* together can completely reverse the phenotype of ectopic *dpp* expression in the wing, whereas the expression of either alone has no effect. We conclude that a complex of TSG and SOG is an efficient antagonist of DPP signalling.

To determine whether Tsg is conserved among other species, we sought and found genes in the database related to *Drosophila* TSG in human, mouse, zebrafish and *Xenopus*. In addition, we found a second *tsg*-related sequence in *Drosophila* (*tsg2*) and obtained a second zebrafish *tsg* (*tsg1*) using degenerate polymerase chain reaction (PCR) methods. The protein products show extensive similarity with about 50% of 202 amino-acid residues matching in all four species (see http://darwin.bio.uci.edu/~marshlab/). The pairs of *tsg* genes in fly and fish are closer to each other than to *tsg* in any other species, suggesting independent gene-duplication events in these two species. We mapped the human, mouse and zebrafish (*tsg1*) genes by a combination of fluorescence *in situ* hybridization (FISH) or radiation hybrid mapping. The mouse gene maps to 17E1.3–E2, a region that is syntenic to 18p11.2–3 where the human homologue resides. In zebrafish, *tsg1* is located at linkage group 24-74.5, which is syntenic to the human locus and indicates that all

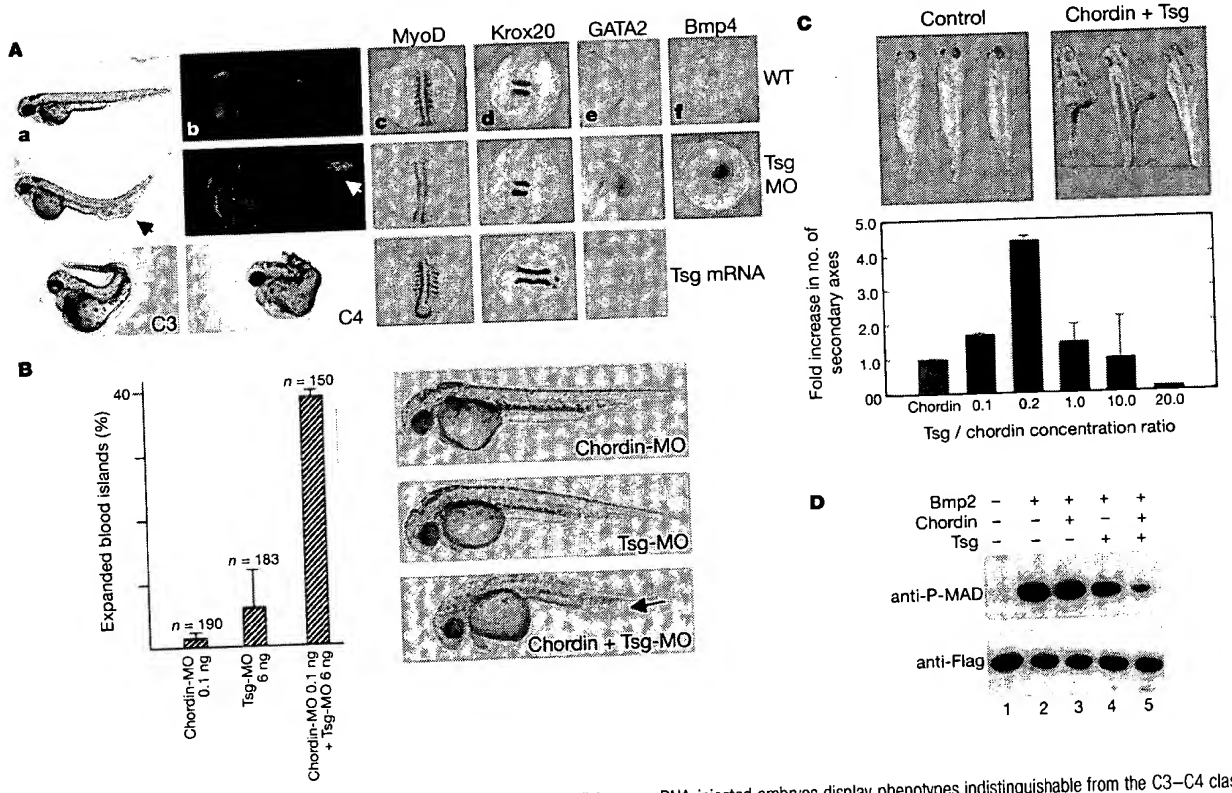

Figure 2 Vertebrate Tsg enhances chordin's antagonist function. **A**, Loss of zebrafish *tsg1* activity ventralizes zebrafish embryos whereas ectopic *tsg1* mRNA has dorsalizing activity. **a**, WT embryo (48 h). **b**, WT embryo injected with 9 ng UroD morpholino oligonucleotides. Blood cells are indicated by red staining. **c–f**, *In situ* hybridization. *myoD* (8 somite stage) (**c**); *krox20* (8 somite stage) (**d**); *GATA2* (22 somite stage) (**e**); and *bmp4* (tail bud view, 3 somite stage) (**f**). Second row as above but injected with 12 ng *tsg1* morpholino oligonucleotide. The filled and open arrows indicate ectopic blood islands (60%, $n = 110$, filled arrow; 60%, $n = 100$, open arrow). Caudal expression of *bmp4* is expanded at the 3 somite stage (48%; $n = 25$), and the blood marker *GATA2* is expanded at the 22 somite stage of development (48%; $n = 21$). Paraxial expression of MyoD is significantly reduced at the 8 somite stage (38%; $n = 33$), and the anterior ectodermal marker Krox20 is moderately reduced at the 8 somite stage (49%; $n = 39$). The third row shows embryos after injection with zebrafish Tsg1 mRNA. At 48 h of development, Tsg1 mRNA-injected embryos display phenotypes indistinguishable from the C3–C4 class of dorsalized mutants[17]. Expression of *myoD* (50%; $n = 8$) and *krox20* (70%; $n = 10$) is also significantly expanded at the 8 somite stage, while *GATA2* and *bmp4* are reduced. **B**, Enhancement of the zebrafish Tsg1 loss-of-function phenotype by sub-inhibitory loss of the chordin gene. Embryos were injected with a low dose of zebrafish Tsg1-MO, chordin-MO, or both, and assayed for blood island expansion (arrow). **C**, Effect of *Xenopus Tsg* on the ability of chordin to block BMP signalling in *Xenopus*. Chordin and Tsg were injected at the ratios indicated. The graph represents the combined results from five experiments. About 200 embryos were injected for each point. On the *y* axis 1.0 for chordin corresponds to 2–16% induction of secondary axes. The chordin mRNA concentration was 5 pg. **D**, Vertebrate Tsg and SOG synergistically inhibit BMP-2 signalling in *Drosophila* S2 cells. The experiment was carried out as in Fig. 1. The mouse protein concentrations were: *bmp2*, 0.2 ng; chordin, 1 μg; and Tsg, 0.5 μg.

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three genes are probably functional orthologues.

The zebrafish *tsg1* gene is expressed uniformly in early embryos, whereas zebrafish *tsg2* is only expressed at later stages (data not shown). Hence, we focused our analysis on zebrafish *tsg1* and used morpholino oligonucleotides[13] to reduce the function of this gene in early zebrafish development. Injection of a *tsg1* morpholino oligonucleotide (*ztsg*-MO) produces a phenotype characteristic of expanded BMP signalling (Fig. 2A)[14,15]. Using morphological criteria and fluorescent red blood cells[13], we found that embryos develop expansions of the ventral fin region that correspond to ectopic blood islands (Fig. 2A, arrowheads), a tissue derived from ventral mesoderm. Injected embryos also show an expansion of *GATA2*, loss of paraxial mesoderm (visualized with the marker *myoD*), and a mild reduction of anterior ectodermal tissues (detected by staining for *krox20*). Caudal expression of *bmp4* is also expanded in these embryos (Fig. 2A), while the anterior ectodermal marker *otx2* is reduced (data not shown). Treated embryos also exhibit an expansion in apoptotic cells ventral to the yolk extension (data not shown), similar to *dino* and *mercedes* mutants[14,16]. Overall, this phenotype is very similar to that of *ogon/mercedes* mutants[14,15] and moderate *chordin* loss-of-function mutants, and represents a modest ventralized phenotype[13,14].

Increasing the level of zebrafish *tsg1* by injecting messenger RNA produced phenotypes characteristic of diminished BMP signalling[17–19] including reduced axial length with loss of ventral fin (Fig. 2A), an expansion of *myoD* and *krox20*, and a reduction in *GATA2*. This is a phenocopy of the C3–C4 class of dorsalized mutant embryos, similar to that of the Snailhouse (BMP7 homologue) and Piggytail mutations[17,19]. Furthermore, the dorsalizing effect of zebrafish Tsg1 mRNA partially reverses the ventralizing effect of *tsg1* (9 ng *tsg1*-MO caused 47 ± 2%, n = 376, ventralized embryos; 9 ng tsg1-MO plus 30 pg Tsg1 mRNA resulted in 19 ± 8%, n = 270, ventralized embryos) suggesting that loss of *tsg1* is responsible for the phenotype. We conclude that loss of *tsg1* leads to embryos with a ventralized phenotype, whereas ectopic expression of *tsg1* leads to a dorsalized embryonic phenotype.

As our *Drosophila* data suggested that one function of TSG is to co-operate with SOG to inhibit BMP signalling, we asked whether the same relationship is true in vertebrates by determining whether a modest reduction of zebrafish *chordin* activity could enhance the effect of a moderate reduction in *tsg1* activity. Sub-inhibitory levels of a zebrafish *chordin* morpholino oligonucleotide and *tsg1*-MO were injected into wild-type embryos, and the effect on ectopic blood island development was scored. These two morpholino oligonucleotides synergistically enhanced blood island expansion (Fig. 2B), supporting the view that both of these gene products co-operatively inhibit BMP signalling. As with the *Drosophila* components, we found that the combination of purified mouse chordin and Tsg was better able to inhibit mouse BMP-stimulated phosphorylation of Mad in S2 cells than either could alone (Fig. 2D).

We also tested for synergy between Tsg and chordin mRNA in *Xenopus* embryos by co-injecting their mRNAs and scoring for enhancement of secondary axis formation[20]. Co-injection of *Xenopus* Tsg and chordin reveals a dose–response optimum. When a sub-inhibitory dose of chordin mRNA is supplemented with increasing levels of Tsg mRNA, the fraction of embryos exhibiting a secondary axis increases up to 4.5-fold over chordin alone at a 1/5 ratio of Tsg/chordin mRNA. However, if the Tsg/chordin ratio is increased to 1:1 or higher, the number of secondary axes is reduced to basal levels and the resulting tadpoles have normal morphology. Injection of 150 pg Tsg alone (the highest concentration of Tsg mRNA used in these experiments) had no effect on embryonic development. Notably, if we increase the level of Tsg relative to chordin in the S2 experiments, we do not see a reversal of the inhibition phenotype (data not shown), suggesting that additional factors probably modulate the *in vivo* response. Taken together, we conclude that, like *Drosophila* TSG, vertebrate Tsg can co-operate with chordin to inhibit BMP signalling.

As a final test of the functional equivalence of the vertebrate and invertebrate *tsg* genes, we expressed the human and mouse genes under the control of the UAS promoter in flies, and injected *Drosophila* TSG mRNA into zebrafish embryos. The phenotype of animals expressing human *TSG* and *Drosophila sog* in wing discs (Fig. 3a) resembles that of *dpp* shortvein alleles[21] and is very similar to that produced by coexpression of the *Drosophila tsg* and *sog* genes (Fig. 3a; see also ref. 9). When injected into zebrafish, *Drosophila tsg* produces a dorsalized phenotype equivalent to that produced by zebrafish *tsg1*, which includes reduced axial length and expansion of *krox20* (Fig. 2A; compare with Fig. 3b) and *myoD* (data not shown).

Our experiments, and those of others[22–24], suggest that Tsg has three molecular functions. First, it can synergistically inhibit Dpp/BMP action in both *Drosophila* and vertebrates by forming a tripartite complex between itself, SOG/chordin and a BMP ligand (Fig. 1B, see also refs 9, 24). Second, Tsg seems to enhance the Tld/BMP-1-mediated cleavage rate of SOG/chordin and may change the preference of site utilization (O.S. and M.B.O., unpublished observations; see also refs 9, 23). Third, Tsg can promote the dissociation of chordin cysteine-rich (CR)-containing fragments from the ligand[24]. Different organisms may exploit each of these properties to different degrees during development depending on the relative *in vivo* concentrations of each molecule. We propose that in *Drosophila* and zebrafish the primary function of Tsg is to form a tripartite complex between itself, Sog/chordin and a BMP ligand. In *Drosophila*, this complex acts to redistribute a limiting amount of DPP, such that activity is elevated dorsally at the expense of being lowered laterally. The net driving force for this redistribution is likely to be diffusion of SOG from its ventral source of synthesis[25].

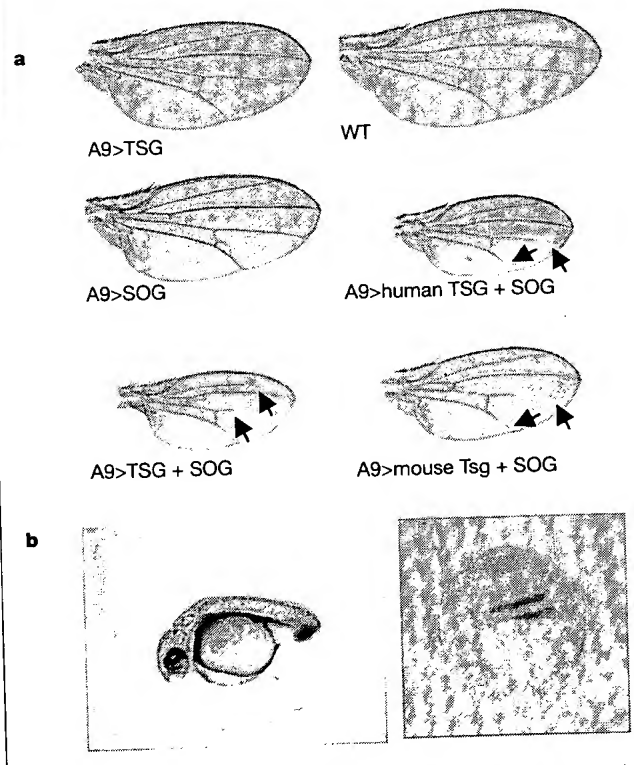

Figure 3 Functional equivalence of Tsg proteins. **a**, The GAL4-A9 driver was crossed to flies at 25 °C (first column) or 29 °C (second column) with the indicated transgenes. Arrows indicate loss of distal vein material. **b**, Injection of 30 pg *Drosophila* Tsg into zebrafish embryos results in a C3-like dorsalized phenotype (54%; n = 22) at 48 h after fertilization (left). Right, embryo injected with 30 pg *Drosophila tsg* exhibiting expansion of *krox20* at the 8 somite stage (compare with Fig. 2a).

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This is consistent with the finding that SOG diffusion is essential for activation of genes such as *race* that require high levels of DPP/SCW signalling (Fig. 1, see also ref. 26). In this model TLD would serve to modulate both the net movement of DPP and its release from the inhibitory complex by cleaving SOG[8,25]. The ability of TSG to enhance the rate of SOG cleavage may also be an important aspect of this model in that it helps ensure the proper timing of these rapid developmental events. It seems unlikely that TSG is needed to remove an inhibitory CR-containing fragment from DPP as the affinity of full-length SOG for DPP in the absence of TSG seems to be low. Likewise, in zebrafish the phenotype of reduced Tsg function is ventralized and not dorsalized as would be predicted if Tsg were primarily needed to release inhibitory CR fragments from ligand. In *Xenopus*, however, perhaps the endogenous levels of full-length chordin and CR fragments are higher than in zebrafish, thereby making the CR displacement activity of Tsg the more important biological function[24]. Determination of the *in vivo* levels of these proteins, along with a more careful analysis of the concentration optima for each type of reaction involving Tsg function, will be required before we can fully understand all of its *in vivo* activities. □

Methods

Isolation of *tsg* clones and gene mapping

Human *TSG* complementary DNA clones (accession numbers AW160804, AA905905, AI222228, AA486291, AI018381, AI379897 and AA758784) were obtained from Research Genetics. One clone (AI018381) was sequenced in its entirety, additional 5′ sequence was obtained from published Est sequences. Mouse *Tsg* cDNA clone (accession number AW258143) was also obtained from Research Genetics. The zebrafish *tsg1* was isolated from an epiboly cDNA library (S. Ekker) using two degenerate primers (5′ primer: 5′-TG(CT)TG(CT)AA(AG)GA(CTAG)TG(CT)(CTA)T-3′ and 3′ primer: 5′-CC(CTG)A(CT)(AG)GA(CT)TC(AG)CA(AG)CA-3′). These primers amplified a 0.5-kilobase (kb) fragement that was used as a probe to identify a 1.2 kb cDNA from a zebrafish epiboly library. We sequenced this clone using standard methods. The human *TSG* locus was mapped against the Stanford G3 hamster–human radiation hybrid panel using primer pairs at the beginning, middle and end of the *TSG* mRNA. This placed human *TSG* between STS markers D18, and D18 within cytogenetic band 18p11.2. The mouse *Tsg* was mapped by FISH using a 16-kb genomic mouse *Tsg* fragment as a probe. The chromosomal assignment and band designation were determined by sequential G-banding to FISH. The zebrafish (*Danio rerio*) *tsg1* gene was mapped to linkage group 24 at 74.5 cM using a mouse–fish radiation hybrid panel[27].

Altering signal peptides

While conducting these studies, we found that the secretion signals of the mammalian and *Drosophila* genes are incompatible with the other species. To circumvent these secretion-related problems, we used PCR to replace the human and mouse signal sequences with the *Drosophila* sequence and also the *Drosophila* signal peptide with the zebrafish sequence (details are available on request).

Production and purification of recombinant proteins

Recombinant proteins SOG-Myc, Tsg-His and Dpp-haemagglutinin (HA) were produced as described[9]. Conditioned medium containing SOG-Myc was applied to a 1 × 10-cm^2 S-Sepharose column (Pharmacia) equilibrated with 100 mM MOPS-Na, pH 6.0 (buffer A). After washing with buffer A containing 300 mM NaCl, the column was eluted with buffer A containing 750 mM NaCl, and the fractions were combined and stored for further use. Conditioned medium containing Tsg-His was applied to a 1 × 10-cm Q-Sepharose column (Pharmacia) equilibrated with 50 mM Tris-HCl, pH 7.5 (buffer B). After washing with buffer B containing 200 mM NaCl, the column was eluted with buffer B containing 500 mM NaCl, and the fractions were combined and applied to a 1 × 4-cm Ni-NTA agarose column (QIAGEN) equilibrated with 100 mM Tris-HCl, pH 8.0 (buffer C). After washing with buffer C containing 1 M NaCl, the column was eluted with buffer C containing 100 mM imidazole. Fractions containing Tsg-His were pooled and dialysed against 50 mM Tris-HCl, 150 mM NaCl, pH 7.4.

Signalling assays

Ten micrograms of Flag-tagged MAD were transfected in S2 cells at 2 × 10^7 cells per dish. After 3 days, the cells were collected and split into 20 samples. One microgram Tsg-His and/or 1.25 μg SOG-Myc (Fig. 1C), or 0.5 μg mouse Tsg-protein C and/or 1 μg chordin-His (R&D Systems) (Fig. 2D) were premixed for 3 h at room temperature (RT) with 10^{-9} M Dpp or 10^{-11} M BMP2 (R&D Systems) and then incubated with S2 cells expressing Flag-Mad for 3 h at RT. The cells were spun down and lysed by 1 × SDS–PAGE buffer. The supernatants were separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membrane was probed with anti-Phospho Mad PS1 antibody at 1/5,000 dilution[5] and anti-Flag M2 antibody (Kodak) at 1/2,000 dilution, followed by incubation in secondory antibody (horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse; Jackson Laboratory) and developed using ECL substrate (Pierce).

Morpholino oligonucleotides

We obtained Morpholino oligonucleotides from Gene Tools, LLC (Corvallis). We selected sequences on the basis of the design parameters recommended by the company. The zebrafish chordin morpholino oligonucleotide was as described[13]. *tsg1*-MO 5′-CTGATG ATGATGATGAAGACCCCAT-3′.

Embryo manipulations and microinjections

Morpholino oligonucleotides were injected as described[13]. For *Xenopus* injections, embryos were obtained by *in vitro* fertilization and cultured as described[28]. Microinjections of mRNA were performed at the 4-cell stage in 0.3 × MMR, 3.5% Ficoll. We determined dorsal–ventral polarity of early cleavage stage embryos using pigmentation differences[28].

In situ hybridization and antibody staining

Hybridization to *Drosophila* and zebrafish embryos was as described[4,29]. The rabbit anti-phospho Mad antibody was a gift from P. ten Dijke and used at 1/2,000 dilution. Staining was visualized using an alkaline phosphatase-coupled secondary antibody (Promega Laboratories).

Received 29 August 2000; accepted 8 January 2001.

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Acknowledgements

We are grateful to K. Cho, D. Greenspan and A. Hemmati-Brivanlou for communication of results before publication and to E. De Robertis for comments on the manuscript. We thank M. Tsang at R&D systems for a gift of purified mouse chordin; D. Greenspan for purified mouse Tsg-C protein; J. Groppe for purified Dpp; P. ten Dijke for the gift of anti-phospho Mad; and E. De Robertis for the *Xenopus Tsg* cDNA clone. We also thank E. Hirsch for assistance with the mouse FISH analysis. This work was supported by NIH grants to J.L.M, M.B.O and S.C.E. O.S. was supported by Nippon Roche K.K. A.P. was supported by a training grant from the NIH. M.B.O. is an Associate Investigator for the Howard Hughes Medical Institute.

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Twisted gastrulation can function as a BMP antagonist

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Bone morphogenetic proteins (BMPs), including the fly homologue Decapentaplegic (DPP), are important regulators of early vertebrate and invertebrate dorsal–ventral development[1–6]. An evolutionarily conserved BMP regulatory mechanism operates from fly to fish, frog and mouse to control the dorsal–ventral axis determination. Several secreted factors, including the BMP antagonist chordin/Short gastrulation (SOG)[7–12], modulate the activity of BMPs. In *Drosophila*, Twisted gastrulation (TSG) is also involved in dorsal–ventral patterning[13–15], yet the mechanism of its function is unclear. Here we report the characterization of the vertebrate Tsg homologues. We show that Tsg can block BMP function in *Xenopus* embryonic explants and inhibits several ventral markers in whole-frog embryos. Tsg binds directly to BMPs and forms a ternary complex with chordin and BMPs. Coexpression of Tsg with chordin leads to a more efficient inhibition of the BMP activity in ectodermal explants. Unlike other known BMP antagonists, however, Tsg also reduces several anterior markers at late developmental stages. Our data suggest that Tsg can function as a BMP inhibitor in *Xenopus*; furthermore, Tsg may have additional functions during frog embryogenesis.

We isolated human Twisted gastrulation (TSG) in a screen for secreted factors, and mouse and *Xenopus* Tsg by low-stringency hybridization using human TSG as the probe. These vertebrate Tsgs have a high sequence homology to each other (more than 80% identical) and are about 30% identical to *Drosophila* TSG at the amino-acid level (data not shown). Tsg is expressed maternally and in all developmental stages in *Xenopus*, and at least from gastrula stages onward in mouse (data not shown). Expression of Tsg is also detected in a variety of adult tissues in both mouse and human (data not shown).

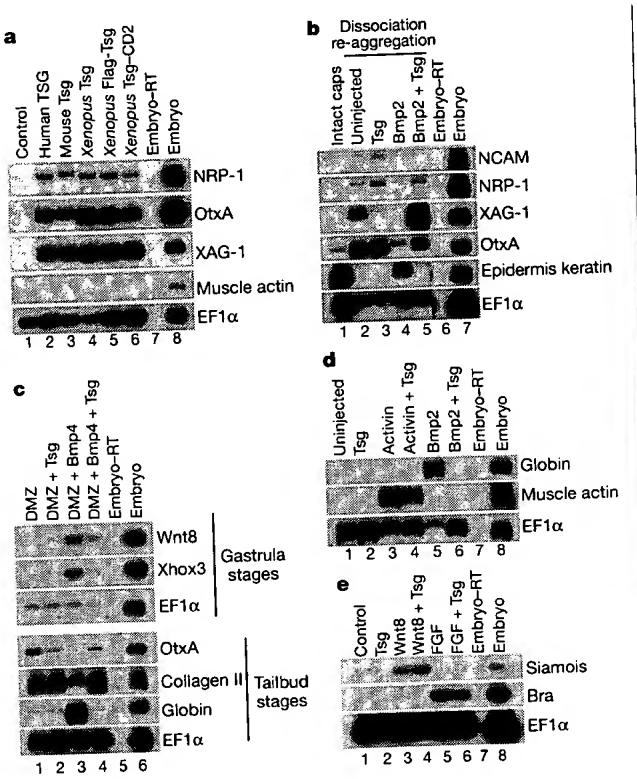

Figure 1 Vertebrate Tsgs inhibit BMP signalling in embryonic explants. **a**, Vertebrate Tsgs induce expression of cement gland and neural markers in animal caps. **b**, *Xenopus* Tsg blocks epidermal induction and neural inhibition by Bmp2 in dissociated animal-cap cells. **c**, *Xenopus* Tsg blocks ventralization of dorsal marginal-zone explants by Bmp4. **d**, *Xenopus* Tsg blocks mesodermal induction by Bmp2, but not activin. **e**, *Xenopus* Tsg does not interfere with the Wnt or FGF signalling. In animal-cap assays, RNAs were injected into animal poles of both cells of 2-cell-stage embryos. Animal caps were dissected at blastula stages (stage 9) and incubated to gastrula (stage 11, **e**) or neurula stages (stage 20, **a**, **b**, **d**). In **b**, the caps were dissociated at blastula stages for 4 h before re-aggregation and incubation to neurula stages, as described[16]. In the marginal-zone assay, RNAs were injected into the two dorsal blastomeres of 4-cell-stage embryos. Dorsal marginal-zone explants were dissected at early gastrula stage (stage 10) and incubated to mid-gastrula (stage 11) or tailbud (stage 28) stages. Xhox3, Wnt8 and globin are ventral markers, whereas OtxA and collagen II are dorsal markers. The weak induction of Wnt8 is not always observed. In **e**, the basic FGF protein (Sigma) was added at 100 ng ml^{-1} to the animal caps at the blastula stages. The amount of RNA injected into the embryos was: 2 ng, all Tsg; 0.5 ng, Bmp2; 0.5 ng, Bmp4; 5 pg, activin; and 50 pg, Wnt8.

To study the function of Tsgs, we first analysed their activities in *Xenopus* ectodermal explants (animal caps). As shown in Fig. 1a, human, mouse and *Xenopus* Tsg induce the cement gland and the neural markers XAG-1, OtxA and NRP-1 with comparable efficiency, suggesting that these vertebrate Tsgs function similarly in *Xenopus*. The induction of cement gland and neural markers in animal caps in the absence of mesoderm is normally associated with inhibition of the BMP signalling[16–18], so we therefore addressed whether Tsg could directly block the activity of BMP. We first examined the effect of Tsg on ventralization of the ectodermal cells by BMPs. As described previously[16], intact animal caps express high levels of epidermal keratin. This expression is suppressed when caps from blastula stages are dissociated for 4 h (Fig. 1b, lanes 1 and

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